Original Article

Characterization of Common β-Thalassemia Major Mutations in Southwest Iran with Respect to Biochemical Parameters, Oxidative Status and Complications

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Abstract

Background & Objective: Beta-thalassemia are among the most common autosomal recessive genetic disorders in Iran, especially in Khuzestan province. Beta-thalassemia exhibits significant phenotype heterogeneity and there are currently more than 200 known mutations in this region. Oxidative stress exacerbates multiple disorders, including thalassemia, an inherited hemolytic anemia caused by globin gene mutations**.** We aim to characterize significant mutations of widespread β-thalassemia in south-western Iran with respect to biochemical parameters, oxidative status and complications of diseases.

Material & method: Forty-five patients, aged between 15-35 years with β-thalassemia major were selected. The patients were receiving regular blood transfusion and chelation therapy and have been previously characterized to bear beta globin gene mutations. The subjects' medical histories were documented by review of previous medical records. We also determined biochemical parameters including glycemic and iron indices, hepatic and renal function tests, oxidative stress markers and levels of advanced glycation end product species (Carboxy methyl lysine and Pentosidin).

Results: The most common mutation was found to be CD36/37(28.9%) followed by IVSII-1, and IVSI-110. Values of iron indices were significantly different in various mutation groups. Carboxy methyl lysine and pentosidine were found to be higher in the β-thalassemia patients with IVSII-1 and IVSI-110, respectively. Also sLOX-1 was found to be significantly higher in IVSI-110 group. Complications of the disease were differently presented in mutation groups and hemochromatosis, hepatomegaly, and diabetes were among the most common problems.

Conclusion: About 72 % of β-thalassemia major cases in southwest Iran result form 3 common mutations with different clinical and laboratory presentations. Molecular genetic testing can be helpful to evaluate the patients' situation.

Keywords: **β**-Thalassemia major; Mutation; Iron overload; Oxidative stress; Khuzestan

Introduction

Thalassemia are a group of inherited disorders of hemoglobin synthesis and the most common monogenetic disease worldwide. It is caused by mutations in the β-globin gene or its promoter, resulting in reduced or absent β-globin synthesis (1,2). Patients with deleterious mutations in both β genes (βT/βT) and imbalanced synthesis of globin chains, display ineffective erythropoiesis and various aspects of anemia (3,4). WHO has estimated that about 1.5% of the world's population might be carriers of β-thalassemia (β/βT) and about 60,000 severely affected infants are born every year (3). In new global health statistics thalassemia affects about 4.5 of every 10,000 live births in the world. (http://www.ironhealthalliance.com/disease-states/thalassemia/ epidemiology-and-pathophysiology.jsp) Iran, with approximately 25,000 β- thalassemia major patients and 2 million carriers, faces a serious prevention problem (5). Khuzestan province is a region with

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the highest prevalence of the beta thalassemia, the most common hemoglobinopathy in Iran (6). More than 200 different mutations with various levels of defect in β-globin gene expression have been identified. Patients can be classified based on the genotypes of β-globin gene cluster (7). In Iranian population, the common mutations have been reported as CD36/37 (- T), IVSII-1(G> A), and IVSI-110 (G> A) (8,9). Patients with β-thalassemia major are characterized by severe anemia in the first year of life and subsequently require regular blood transfusions for survival (1,10). Iron overload as a severe complication develops in the patients due to frequent transfusions and lead to accumulation of iron in heart, liver, pancreas and endocrine organs. This causes oxidative stress and serious damage to these vital organs (11). When transferrin is saturated with iron, excess iron begins to circulate as non-transferrin bound species (NTBI) and free iron initiates redox reactions to produce reactive oxygen species (ROS) and lipid peroxidation (12,13). The production of ROS by iron is mainly through the Fenton reaction, which eventually forms hydroxyl radicals from superoxide or hydrogen peroxide (14). Oxidative stress makes an important contribution to numerous pathologies including cancer, cardiovascular and degenerative diseases (11).

Advanced glycation end-products (AGEs) are formed by the reaction of reducing sugars with free amino groups of proteins or amino acids (15). Interaction of AGE with their receptors (RAGE), plays an important role in the pathogenesis of numerous diseases, including diabetic complications, atherosclerosis, hypoxia/reoxygenation injury, and aging (16,17). Evidence suggests that AGEs are involved in a defective cycle of free radical generation. Different types of AGEs are known, depending on the compound from which they originate (18). CML is formed during copper-catalyzed oxidation of polyunsaturated fatty acids in the presence of protein. Therefore, CML is suppressed by desferrioxamine, iron chelator or anti oxidative enzyme (19). Thus, between the signs caused by oxidative stress are elevated intravascular (within the blood vessels) and extravascular (mainly spleen and liver) hemolysis, inadequate RBC (erythropoiesis) development and dysfunction of essential organs such as heart and liver and endocrine system (20).

This research aims to characterize the β-thalassemia mutations in a population of β-thalassemia major patients of Khuzestan province and to investigate levels of iron overload, oxidative stress, advanced glycation end-products and biochemical parameters in β-thalassemia patients with different common β-globin gene mutations. Common adverse complications of the disease are also described with regard to the different mutation types.

Materials & Methods

Subjects

Patient population included 45 beta-thalassemia subjects aged 15-35 years. Patients were recruited from Shafa hospital in Ahvaz, Iran. All patients have been previously characterized to bear β-globin gene mutations. Blood samples were collected from the patients just before the transfusion. The patients received approximately 15 ml of packed red blood cells per kilogram body weight at each transfusion once a month in to maintain the Hb level higher than 10 g/dl. The patients were under irregular chelation therapy with Deferoxamine or Deferiprone. After enrollment, medical histories of the subjects were documented by a review of previous medical records with regard to the complications of the disease. A medical record was also conducted by the research coordinator at the patient's centre, which included documentation of transfusion and chelating history and recent endocrine laboratory values. The study protocol was approved by the local ethics committee at the Ahvaz Jundishapur University of Medical Sciences (IR.AJUMS.REC.1395.76), and have been performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments. Informed consent was obtained from all participants before including them in the study.

Clinical samples were collected from venous blood following 12 hours fasting in two different tubes (with and without EDTA as anticoagulant). Plasma was separated by centrifugation at 3500 x g for 10 minutes and aliquots of plasma were stored at –70°C immediately after separation.

Biochemical analysis

Biochemical parameters, including plasma glucose, iron, transferrin, ferritin, and liver function tests including alanine aminotransferase

(ALT) and aspartate aminotransferase (AST), renal function test, including creatinine and urea were analyzed using an automated analyzer (auto-analyzer BT 3000, Italy). Total Iron-binding Capacity (TIBC) was estimated by transferrin levels. Human transferrin forms a precipitate with a specific antiserum, whose turbidity is determined metrically at 340 nm. TIBC was then calculated according to the following formulae: TIBC (µg/dl) =Transferrin (mg/dl) X 1.25

As an estimate of free iron or non–transferrin-bound iron (NTBI), Iron excess of TIBC (IET) was calculated according to the following formulae: IET (µg/dl) = [Fe] (µg/dl) - TIBC (µg/dl)

Direct enzymatic assay was used to measure HbA1c in whole blood samples (Auto-analyzer Hitachi 912, Japan).

Oxidative stress analysis

Thiobarbaturic acid reactive substances (TBARS) were measured in serum samples by a colorimetric reaction with thiobarbituric acid (TBA) at high temperature during 45 min in manual. TBARS adduct was extracted by n-butanol and its absorbance was read at 532 nm and quantified by reference to a calibration curve of tetraethoxypropane (Sigma), submitted to the same TBA reaction.

Plasma sLOX-1 was quantified using a kit (EASTBIOPHARM ELISA catalog no. Ck-E91390, USA), which was a solid-phase two-site enzyme immunoassay based on the direct sandwich technique, in which two monoclonal antibodies were directed against separate antigenic determinants on soluble ox-LDL receptor. (Reference range for sLOX-1: 0.05ng/ml-30ng/ml)

Measurement of advanced glycation end-products species

Carboxy methyl lysine (CML) and pentosidine (PTD) protein adducts present in the serum samples were measured by enzyme-linked immunosorbent assay using a commercial kit (EASTBIOPHARM, catalog No.Ck-E90218, CK-E10640) based on the manufacturer's protocol. (Reference range for PTD: 0.05ng/ml-20ng/ml and Reference range for CML: 20ng/ml-3000ng/ml)

Statistical analysis

SPSS version 22.0 was used for data analysis. Quantitative data were presented as mean ± standard deviation (SD). Following normality analysis of variables in β-thalassemia and control groups, data from different groups of mutation types were compared using analysis of variance (ANOVA) test followed by post hoc Tukey test for multiple comparisons. For all tests, $P < 0.05$ was considered significant. Contingency tables and percentage were used to describe quantitative parameters.

Results

Subjects characteristics

The patient group had the mean age of 24±4 (15-35) years (24 males and 21 females). The frequency of mutations for Arab and Persian ethnicity in β-thalassemia major patients was shown in Table 1.

Table 1. Frequencies of common mutations recorded in β-thalassemia patients

		CD 36/37	IVS II-I	IVS I-110	other	p-value
Gender	male	14 (15.6%)	12 (13.3%)	8 (8.85%)	14 (15.6%)	-
	female	12 (13.3%)	10 (11.2%)	8 (8.85%)	12 (13.3%)	-
Ethnicity	Arab	18 (20%)	12 (13.3%)	8 (8.85%)	10 (11.2%)	-
	Persian	8 (8.9%)	10 (11.2%)	8 (8.85%)	16 (17.7%)	-
Total		26 (28.9%)	22 (24.5%)	16 (17.7%)	26 (28.9%)	-
Age (year)		24.19±4.40	25.50±6.45	24.66±5.16	25.20±6.53	0.923
BMI (kg/m^2)		20.38±2.17	20.94±3.11	20.41±1.35	19.43±3.71	0.766
Volume of transfusion (ml)		509.61±66.36	512.50±35.35	600.00±122.47	520.00±44.72	<0.05[a]
Duration of transfusion (year)		21.53±4.52	23.50±6.54	23.16±4.79	22.20±6.90	0.773

[a] Indicates a significant difference between groups (P<0.05).

The most frequent mutation was found to be CD36/37 (28.9% of the cases) in which most of patients had Arab ethnicity (9 patients) compare to 4 Persian patients. This was not true for the rest of mutation types in which different ethnicities had almost equal frequencies. Mutation types of CD36/37, IVS II-I and IVS I-110 together accounted for 71.1 % of cases with β-thalassemia major. In addition patients with various mutation types were not significantly different with regard to their BMI or duration time of receiving blood transfusions. However, the volume of transfused blood was significantly different in patients with various mutation types.

Results of biochemical measurements

As shown in Table 2, fasting plasma glucose (FPG) in patients with CD36/37 and IVS II-1 mutation was higher than the other mutation types. The FPG levels in these two groups were higher than 126 mg/dl, the cut-off point of diabetes mellitus recognition. HbA_1c levels showed similar pattern to that of FPG in different groups, but the differences were not statistically significant.

Levels of liver enzymes AST and ALT as well as urea and creatinine did not show significant differences between mutation groups.

Variables of iron status including plasma iron, ferritin, TIBC and IET displayed different levels in mutation types. Patients with CD36/37 mutation had lower plasma iron than patients with IVS I-110 or IVS II-1 mutations ($P< 0.05$). Ferritin was higher in CD36/37 group than IVS II-1 group ($P< 0.05$). TIBC was higher in IVS I-110 than other groups and IET was significantly higher in IVS II-1 than CD36/37 group ($P< 0.05$).

Table 2. Values of biochemical parameters in β-thalassemia patients with common mutations.

	CD36/37	IVS II-I	IVS I-110	Other	p- value
Glc(mg/dl)	140.46±97.51	130.37±102.43	119.33±23.14	123.00±23.90	0.940
HbA1c (%)	7.26±2.22	7.36±1.86	6.73±0.82	6.52±0.81	0.803
AST(U/l)	49.69±26.98	45.00±28.94	54.83±40.61	59.50±35.60	0.856
ALT(U/l)	47.56±37.72	36.75±27.50	51.50±45.33	54.20±23.73	0.814
Creatinine(mg/dl)	0.61±0.13	0.63±0.12	0.71±0.10	0.59±0.16	0.396
Urea(mg/dl)	25.72±6.91	27.60±8.70	29.93±8.00	29.96±3.73	0.443
Iron(µg/dl)	185.53±49.49	238.50±53.74	253.16±39.78	206.00±26.20	<0.05 [a]
Ferritin(ng/ml)	4991.57±3372.46	1578.00±1157.62	4548.33±2249.49	5747.60±4259.38	<0.05 [a]
TIBC(µg/dl)	158.88±33.33	165.62±16.28	208.33±20.72	162.00±8.60	<0.05 [a]
IET(µg/dl)	25.50±31.87	68.75±59.88	53.83±29.86	44.00±19.19	<0.05 [a]

[a] Indicates a significant difference between groups ($P<0.05$).

Comparison of oxidative stress markers and AGEs in mutation groups

Carboxy methyl lysine (CML) and pentosidine were found to be higher in the patients with IVS II-1 and IVS I-110, respectively. However, only CML showed a significant difference (Table 3). In addition sLOX-1 showed significantly higher levels in IVS I-110 group than CD36/37 (P< 0.05). A comparison of the oxidative stress and AGEs levels in mutations types is shown in Table 3.

observed in the patients. The most frequent problem found to be hemochromatosis. These complications were presented differently in mutation groups. Descriptive results of the frequencies of these problems are shown in Table 4. Hemochromatosis, Hepatomegaly in IVS II-I were more common in hemochromatosis than in other mutations. In both CD36/37 and IVS II-I mutations, diabetes and heart disease were the same, but in IVS I-110, bone disease affected more patients. The prevalence of

Table 3. Levels of oxidative stress markers and AGEs in β-thalassemia patients with common mutations.

	CD36-37	IVS II	IVS I-110	Other	p- value
CML(ng/ml)	2096.38±1448.80	3547.59±1169.46	2554.10±1552.77	1430.96±732.41	<0.05[a]
PTD(ng/ml)	56.04±54.17	77.56±64.73	88.73±57.72	52.39±47.78	0.505
MDA(µg/dl)	8.88±3.99	8.00±4.00	11.50±5.78	12.20±6.01	0.238
s-LOX(ng/ml)	16.38±5.98	18.04±7.31	24.22±4.03	17.12±8.53	<0.05[a]

Table 4. Frequencies of major complications in β-thalassemia patients with common mutations

	CD36/37 n =26	IVS II-I n =22	IVS I-110 n =16	Other n =26
Hemochromatosis	10 (38.5%)	12 (54.5%)	8 (50.0%)	8 (30.7%)
Hepatomegaly	6 (23.1 %)	8 (36.3%)	4 (25.0%)	6 (23.1%)
Diabetes	6 (23.1%)	6 (27.3%)	2 (12.5%)	4 (15.4%)
Heart disease	4 (15.4%)	4 (18.2%)	2 (12.5%)	4 (15.4%)
Bone disease	4 (15.4%)	2 (9.1%)	6 (37.5%)	4 (15.4%)
Hypothyroidism	2 (7.7%)	0 (0%)	0 (0%)	0 (0%)
Hypogonadism	2 (7.7%)	0 (0%)	0 (0%)	0 (0%)

Frequency of common β-thalassemia complications in mutation groups

Beta-thalassemia complications including hemochromatosis, hepatomegaly, diabetes mellitus, heart disease, bone disease, hypothyroidism, and hypogonadism were

hypogonadism and hypothyroidism was not meaningful.

Discussion

In the present study, we investigated biochemical parameters, oxidative stress

markers, level of advanced glycation end-products and frequency of β-thalassemia complications in different groups of β-thalassemia major patients with common mutation types.

Most of β-thalassemia cases are due to point mutations and large deletion mutations are found scarcely in patients (3). Prevalence rate of mutations in different races and ethnic groups is varied, and in each geographical area some mutations are more common than others. For example in Sardinia, Italy, 95.7% of β-thalassemia patients have CD 39 C>T mutation (21). In Khorasan province of Iran, Jaripour et al defined IVS-I-5 as the most common mutation while codons 8/9 are recognized as the third most common mutation (22). In another study in Hamadan province of Iran has shown three forms of mutations, including IVS-II-1 (G > A), 8/9 codons, and 36/37(–T) codons, which constituted more than 50.0 percent of the mutations identified (23). In this study, CD 36/37 (-T) was the most prevalent mutation in Ahvaz Shafa hospital with a frequency of 28.9%, followed by IVS II-1and IVS I-110. These findings are in line with a pervious study conducted in Khuzestan province that reported CD 36/37 (-T) with a frequency of 20.54% and IVS II-1 (G>A) with a frequency of 20.01% mutations as the most prevalent types (6,9). Besides, in a study in Southwest of Iran Nezhad et al. found that the occurrence of β-thalassemia IVSII-1 (G> A) mutation had the highest incidence (24).

Transfusion as a therapy has extended and improved the quality of life in patients with β-thalassemia. This treatment leads to chronic iron overload. Transfusion-dependent patients, in the absence of chelation therapy, develop progressive accumulation of iron in tissues, which initiates oxidative damage to vital organ such as liver, heart and endocrine glands. Elevated levels of FPG and HbA1c in the patients compared to normal ranges confirm the previous reports of impaired glucose homeostasis and pancreas damage (25). However, the HbA1c test may be impaired by other causes, such as abnormal hemoglobin, anemia and certain medications (26). El-Samahy et al. have shown chelation conformity plays an important role in the incidence of glycemic disorders (27).

Elevated transaminases in β-thalassemia may be related to hemolysis process in addition to hepatic damage. Decreased activity of cytochrome c oxidase and interruption of mitochondrial respiration have been reported in β-thalassemia patients. In addition, the liver function damage may contribute to iron overload and deteriorates the harmful situation (28–30).

Increased serum creatinine and uric acid concentrations in β-thalassemia patients can be attributed to perturbation of kidney function and renal tubules damage which is in accordance with previous findings (31,32). These consequences and relevant laboratory parameters were not focused according to different mutation types in previous studies.

In this study, we found higher levels of oxidative stress and AGEs in patients with IVS II-1 and IVS I-110 mutations, which indicate more severe outcomes of these mutations. Oxidative stress in β-thalassemia is due to the key redox reactions of hemoglobin that take place in the presence of hydrogen peroxide (H_2O_2) and superoxide anion radicals (O_2^-). Iron reacts with O_2 species through the Fenton and Haber–Weiss reactions to form cytotoxic hydroxyl radicals (33). Ehteram et al. reported an increase in prooxidant-antioxidant balance (PAB) and hs-CRP in thalassemic patients which might be involved in the pathological consequences of the disease (34). In addition, in the absence of β-globin chain, excessive α-chains undergo self-aggregation. Increased hemolysis due to this abnormal hemoglobin releases more iron and deteriorates the condition (11).

Lipid peroxidation resulting from Non-transferrin bound iron (NTBI) disrupts cell membrane integrity and cellular function

that results in an uncontrolled expansion of the labile plasma iron (LPI) that is not matched by the sequestering capacity of ferritin. Consequently, oxidative stress due to the increased availability of the catalytically active NTBI is a likely cause for much of the organ damage associated with chronic transfusion (33,35).

Smirnova et al. reported that oxLDL reduced nitric oxide (NO) production and upregulated LOX-1 in endothelial cells, as a result of increased oxLDL internalization and subsequent production of reactive oxygen species (36). We observed increased level of sLOX-1 in patient with higher iron overload. Increased oxidative stress upregulates LOX-1 expression in vascular endothelium, and may probably enhance cleavage of sLOX-1 from the cell-surface. These events may cause the adverse cardiac effect and heart diseases in β-thalassemia (37). In our study, patient with different mutation types showed different sLOX-1 levels and the highest level was found in the IVS I-110 mutation.

Many studies have examined the effect of oxidative stress in diabetic patients (38,39). Reactive oxygen species (ROS) and advanced glycation end products (AGEs) are key modulators of diabetes complications (40). Based on our results, impaired glucose homeostasis and diabetes were common problems in β-thalassemia and this could result from excessive iron, oxidative stress and increased AGE levels.

Blood transfusion is a clinical phenotype that changes the phenotype in ß thalassemia patients. A wide clinical spectrum ranging from type of mutation in the β gene as a primary modifier, a secondary modifier that results in the progress of the balance ratio of α and β (41). Various complications are associated with β-thalassemia such as transfusion-transmitted infections, adverse effects of iron overload, toxicity of iron chelation therapy, and bacterial infections. Hypogonadism, hypothyroidism, diabetes mellitus, low bone mass, and hypoparathyroidism are common in young adults with β-thalassemia major (42). Recent studies showed that impairments in osteoblast activity and deferoxamine toxicity are the major causes of osteopenia/osteoporosis in β-thalassemia and enhanced activation of osteoclasts is the contributing factor (43,44). We acknowledge details concerning the history of the patients which have not been mentioned in their documents in some cases, so the information was not consistent and caused difficulty in interpretation.

Iron overload leads to increased oxidative stress and inflammation (45). Tsay et al, showed that ineffective erythropoiesis of thalassemia has been associated with increased serum inflammatory cytokines (46). On the other hand, the mechanism of iron overload in β-thalassemia and hemochromatosis is quite complex and the exact role of ineffective erythropoiesis in regularly transfused patients is unclear (33).

Plasma levels of AGEs were increased in IVS II-1 and IVS I-110 mutations in β-thalassemia major patient. Production of AGEs is enhanced in increased oxidative conditions and interaction of AGEs with their receptors (RAGEs), activates intracellular reactive oxygen species (ROS) production by protein kinase C (PKC)-dependent activation of NADPH oxidase (47). Our previous study showed that plasma levels of CML and pentosidine (PTD) increased in the patients with β-thalassemia major. We also found that and serum PTD was positively correlated with markers of iron overload such as serum ferritin, iron (48). Chen et al. in their recent study demonstrate that a high dosage of iron supplementation may affect iron-AGE-RAGE pathways (49).

As it has been focused in a previous review, certain polymorphisms and mutations in beta-thalassemia patients could dictate the severity of symptoms as well as their onset (50). The genetic basis of thalassemia and the mixture of genotypes and phenotypes shall provide an accurate description of phenotype and the factors that influence them (51). In

addition, Sajadpour et al. discovered that certain polymorphisms in various genome sites and beyond the b-globin gene cluster can increase the development of Hb F-containing RBCs and reduce the symptoms of the disease (52). Accordingly, the present study characterized the common mutations of β-thalassemia in southwest Iran with regard to clinical and biochemical manifestations and can provide an insight into the prognosis for individual patients with different mutations.

Conclusion

Most of the β-thalassemia major cases in southwest Iran result from CD36/37, IVS II-1, and IVS I-110 mutations in β-globin gene. Patients with different mutations show different clinical manifestations and laboratory measures. Thus, molecular genetic evaluations can help to predict the prognosis and better management of patients.

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Conflicts of Interest

The authors declare that they have no conflict of interests.

Ethical Approval

Human and animal rights The study has been approved by the appropriate local ethics committee at the Ahvaz Jundishapur University of Medical Sciences (IR.AJUMS.REC.1395.76) and has been performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Ethical standard All procedures performed in studies involving human participants were in accordance with the ethical standards of the Ahvaz Jundishapur University of Medical Sciences research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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